

## EXPERIMENTAL STUDIES

# Increased Expression of Extracellular Signal-Regulated Kinase and Angiotensin-Converting Enzyme in Human Atria During Atrial Fibrillation

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- OBJECTIVES** The purpose of this study was to determine whether atrial expression of the extracellular signal-regulated kinases Erk1/Erk2 and of the angiotensin-converting enzyme (ACE) is altered in patients with atrial fibrillation (AF).
- BACKGROUND** Recent studies have demonstrated that atrial fibrosis can provide a pathophysiologic substrate for AF. However, the molecular mechanisms responsible for the development of atrial fibrosis are unclear.
- METHODS** Atrial tissue samples of 43 patients undergoing open heart surgery were examined. Seventeen patients had chronic persistent AF ( $\geq 6$  months; CAF), 8 patients had paroxysmal AF (PAF) and 18 patients had no history of AF. Erk expression was analyzed at the mRNA (quantitative reverse transcription polymerase chain reaction), the protein (immunoblot techniques) and atrial tissue (immunohistochemistry) levels. Erk-activating kinases (MEK1/2) and ACE were analyzed by immunoblot techniques.
- RESULTS** Increased amounts of Erk2-mRNA were found in patients with CAF ( $75 \pm 20$  U vs. sinus rhythm:  $31 \pm 25$  U;  $p < 0.05$ ). Activated Erk1/Erk2 and MEK1/2 were increased to more than 150% in patients with AF compared to patients with sinus rhythm. No differences between CAF and PAF were found. The expression of ACE was three-fold increased during CAF. Amounts of activated Erk1/Erk2 were reduced in patients treated with ACE inhibitors. Patients with AF showed an increased expression of Erk1/Erk2 in interstitial cells and marked atrial fibrosis.
- CONCLUSIONS** An ACE-dependent increase in the amounts of activated Erk1/Erk2 in atrial interstitial cells may contribute as a molecular mechanism for the development of atrial fibrosis in patients with AF. These findings may have important impact on the treatment of AF. (J Am Coll Cardiol 2000;35:1669–77) © 2000 by the American College of Cardiology

Atrial fibrillation (AF), the most common arrhythmia in clinical practice, is a potential cause of thromboembolic events (1,2). Several studies have shown that AF induces significant changes of the electrophysiologic properties of atrial myocytes and causes alterations in the structure of the atrial tissue (3–7). In contrast to most AF animal models, patchy necrosis or fibrosis exist in atrial tissue of patients with idiopathic AF (8). Whether atrial fibrosis induces AF or is a consequence of AF is still unknown. Li et al. (9) showed that atrial fibrosis in the failing animal heart

provides a pathophysiologic substrate for AF. Atrial fibrosis was associated with circumscribed electrophysiologic inhomogeneities of the atria allowing the induction of prolonged episodes of AF. The development of atrial fibrosis, therefore, may explain the occurrence of AF in patients with structural heart disease. Alterations of the atrial tissue architecture may also contribute to the mechanical dysfunction of the atria (6,7,10).

The molecular basis for the development of atrial fibrosis in patients with AF is still a matter of debate. Its understanding, however, could have an important therapeutic impact. The purpose of our study was to assess the role of extracellular signal-regulated kinases (Erk1 and Erk2) and its activating mechanisms in patients with and without AF.

Erk1 and Erk2 belong to the subfamilies of mitogen-activated protein kinases (MAPK) (11,12). Activation of the Erk pathway by G protein-coupled receptor agonists

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## Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
AF	=	atrial fibrillation
Ang II	=	angiotensin II
CAF	=	chronic persistent AF $\geq 6$ months
Erk	=	extracellular signal-regulated kinase
JNK	=	c-Jun N-terminal kinase
MAPK	=	mitogen-activated protein kinase
MEK	=	Erk-activating kinase
PAF	=	paroxysmal AF
RT-PCR	=	reverse transcription polymerase chain reaction
SDS	=	sodium dodecyl sulfate
SR	=	sinus rhythm

like angiotensin II (Ang II) induces cellular differentiation processes and an activation of fibroblasts that causes the development of interstitial fibrosis (11–14). Therefore, it is important to find out whether AF is associated with an activation of the erk pathway in the human atrium. Atrial expression and activation of Erk were studied at the mRNA, the protein and tissue levels in patients with chronic or paroxysmal AF. The expression of phosphorylated Erk-activating kinases (MEK1/2) and the atrial angiotensin-converting enzyme (ACE) was also evaluated.

## METHODS

**Patients.** All experiments were performed in compliance with the relevant laws and institutional guidelines. After written informed consent, right atrial appendages were obtained from patients undergoing cardiac bypass surgery or valve replacement (mitral valve and/or aortic valve replacement). Appendages were obtained from 17 patients with chronic AF (defined as persistent AF  $\geq 6$  months; CAF), 8 consecutive patients with documented episodes of paroxysmal AF ( $3 \pm 2$  AF episodes per month; PAF) and from 18 patients without a history of AF. Four patients with PAF were in sinus rhythm and the remainder had AF at the time of surgery. All 43 patients were in New York Heart Association class II. None of them had received amiodarone prior to surgery. The clinical characteristics are shown in Table 1. Expression and activation of protein kinases (MEK1/2 and Erk1/Erk2) as well as expression of the ACE were analyzed in matched groups of patients (age, severity of cardiac and noncardiac diseases, ACE inhibitor therapy) with sinus rhythm (SR) and CAF. In patients with PAF, the amounts of activated MEK1/2 as well as Erk1/Erk2 expression/activation were determined.

**RNA-isolation.** Samples of human atrial appendages were obtained during the surgical procedures and rapidly frozen in liquid nitrogen and stored at  $-192^{\circ}\text{C}$  for further analysis. Total RNA was prepared as described by Chomczynski and Sacchi (15). About 200 mg of tissue was homogenized on ice in 2 ml of TRIZOL (Gibco BRL; Karlsruhe, Germany)

Table 1. Patient Characteristics

	SR	PAF	CAF
Subjects (n)	18	8	17
Age (yr)	$63 \pm 10$	$67 \pm 10$	$66 \pm 6$
Gender (m/f)	12/6	7/1	12/5
History AF (mo)	—	$10 \pm 6$	$53 \pm 40$
CAD (%)	67 (12)	63 (5)	59 (10)
MI (%)	33 (6)	50 (4)	29 (5)
AVD (%)	28 (5)	25 (2)	24 (4)
MVD (%)	22 (4)	13 (1)	18 (3)
LVEF (%)	$54 \pm 15$	$47 \pm 15$	$55 \pm 12$
LAD (cm)	$4.3 \pm 0.8$	$4.6 \pm 0.2$	$5.0 \pm 0.3^*$
Hypertension (%)	44 (8)	50 (4)	47 (8)
Diabetes (%)	33 (6)	25 (2)	35 (6)
ACE inhibitors (%)	61 (11)	75 (6)	59 (10)
Beta-adrenergic blocking agents (%)	44 (8)	38 (3)	41 (7)
Calcium antagonists (%)	28 (5)	25 (2)	41 (7)
Digitalis (%)	22 (4)	38 (3)	47 (8)

\* $p < 0.05$  versus sinus rhythm.

ACE = angiotensin-converting enzyme; AF = atrial fibrillation; AVD = aortic valve disease requiring valve replacement; CAD = coronary artery disease; CAF = chronic AF; LAD = left atrial diameter; LVEF = left ventricular ejection fraction; MI = history of myocardial infarction; MVD = mitral valve disease requiring valve replacement; PAF = paroxysmal AF; SR = sinus rhythm; numbers in parentheses represent the absolute number.

using an UltraTurrax. After the addition of 400  $\mu\text{l}$  of chloroform, the samples were vortexed and centrifuged at  $4^{\circ}\text{C}$  and 14,000  $g$  for 15 min. Total RNA was precipitated from the aqueous phase by the addition of 1 volume of ice-cold isopropanol. The RNA was dissolved in 0.1% sodium dodecyl sulfate (SDS) and reprecipitated by the addition of 1/10 volume 5 mol/liter ammonium acetate, at a pH of 4.8, and 2.5 volumes of ice-cold ethanol. Contaminating DNA was removed by DNase I digestion (Boehringer Mannheim; Mannheim, Germany; 20 U/50  $\mu\text{l}$  reaction, 30 min at  $37^{\circ}\text{C}$ ). Subsequently, RNA was subjected to a second round of purification by means of the RNeasy Mini Kit (Qiagen; Hilden, Germany), and the resulting RNA was quantified spectrophotometrically using a GeneQuant (Pharmacia LKB, Freiburg, Germany). The RNA was aliquoted and stored ethanol-precipitated at  $-80^{\circ}\text{C}$  until use.

**Reverse transcription.** In each case, 1  $\mu\text{g}$  of total RNA was transcribed in a final volume of 20  $\mu\text{l}$  by 20 U of AMV reverse transcriptase (Stratagene; Heidelberg, Germany) in the supplied buffer with the addition of 0.5 mmol/liter dNTP, 10 mmol/liter random hexanucleotides (Boehringer Mannheim) and 50 U of placenta RNase inhibitor (Ambion; Austin, Texas) during a 1-h incubation at  $37^{\circ}\text{C}$ . The enzymes were inactivated by a 10-min incubation at  $65^{\circ}\text{C}$ , and the reaction mixture was kept frozen at  $-70^{\circ}\text{C}$  until enzymatic amplification.

**Quantitative polymerase chain reaction (PCR).** Quantitative PCR was performed in 10- $\mu\text{l}$  reaction capillaries using the Lightcycler LC24 (Idaho Technology; Boston,

Massachusetts). For the determination of Erk2 mRNA amounts, a typical 10- $\mu$ l reaction mixture consisted of 1 $\times$  reaction mixture with bovine serum albumin (Idaho Technology), 3 mmol/liter  $MgCl_2$ , 200  $\mu$ mol/liter dNTPs, 0.4 U InViTaq-polymerase (InViTec; Berlin, Germany), 0.2  $\mu$ l of a 1:1,000 dilution of SYBR Green I (Molecular Probes; 1  $\mu$ l cDNA and 0.5  $\mu$ mol/liter of the Erk2-specific primers Erk2-US (5'-CATCGCCGAAGCACCATTCAAG) and Erk2-DS (5'-GATAAGCCAAGACGGGCTGGAG). Initial denaturation at 95°C for 10 s was followed by 40 cycles with denaturation at 95°C of 0.1 s, annealing at 65°C for 3 s, and elongation at 72°C for 16 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR product, was read at the end of each elongation step.

**Western blotting.** About 200 mg of tissue was homogenized directly in 2 $\times$  RotiLoad (Roth; Karlsruhe, Germany) using an UltraTurrax. The homogenate was cleared by ultracentrifugation at 4°C, 100,000 g, 30 min. Protein contents were determined by using an assay kit (Protein Assay Kit; Sigma; Heidelberg, Germany). After 5 min, boiling samples (300  $\mu$ g each) were applied onto precast 4% to 12% gradient SDS-polyacrylamide gels (NuPAGE; Novex Electrophoresis; Frankfurt a.M., Germany) and separated at 300 V, 15 mA constant using MOPS-SDS running buffer (Novex). Proteins were transferred onto nitrocellulose membrane BAS-85 (Schleicher & Schüll; Goslar, Germany) by means of a semidry blotter (Bio-Rad, Munich, Germany) (25 V, 2 h, 50 mmol/liter Tris-borate-buffer, pH 9.0). Membranes were blocked by overnight incubation in 7.5% (w/v) milk powder in phosphate-buffered saline solution. The following primary antibodies were used for immunodetection: rabbit anti-Phospho-p44/p42 MAP kinase (Thr202/Tyr204) polyclonal, purified Ig (PhosphoPlus p44/p42 Antibody Kit; New England Biolabs; Schwalbach, Germany); rabbit anti-MEK1/2 polyclonal, purified Ig (PhosphoPlus MEK1/2 Antibody Kit; New England Biolabs); mouse anti-ACE monoclonal antibody, IgG1 (Chemicon; Hofheim, Germany). Goat-anti-rabbit-POD or goat-anti-mouse-POD (New England Biolabs) and "SuperSignal West Dura Extended Duration Substrate" (Pierce) were used for chemiluminescence detection. The resulting images were densitometrically quantified by using software (RFLP-Scan software; MWG Biotech; Ebersberg, Germany). The mean relative absorption units of the group with sinus rhythm were set as control and compared with the corresponding means of the AF groups.

To avoid artificial variations of individual blots, protein determination, SDS-polyacrylamide gel electrophoresis, blotting, immunostaining and chemoluminescence detection were performed simultaneously in the same batch of reagents. Densitometric quantification for comparison of the different groups was done only on blots processed equally and exposed on the same X-ray film.

**Histochemistry and immunohistochemistry.** Histochemistry and immunohistochemistry were performed in a total of 22 tissue specimens. Ten specimens were from patients with sinus rhythm, eight from patients with CAF and the remaining four samples were obtained from patients with PAF. Tissue samples were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin/eosin and Masson trichrome. Immunostaining was performed with a polyclonal antibody, as specified above, directed against phosphorylated Erk1/Erk2 (dilution 1:500). Prior to immunostaining, the specimens were pretreated with 10 mmol/liter EDTA (2  $\times$  10 min, 450 W microwave oven). Immunoreaction was visualized by the avidin-biotin-complex method applying a Vectastain ABC-alkaline phosphatase-kit (CAMON; Wiesbaden, Germany). Neufuchsin served as chromogen. The specimens were counterstained with hematoxylin. The specificity of immunostaining was controlled by omitting the primary antibody.

**Statistical analysis.** Differences in the amounts of Erk2-mRNA, activated MEK1/2 and activated Erk1/Erk2 among the three groups of patients were evaluated using one-way analysis of variance. A *t* test with Bonferroni correction was used to evaluate the significance of differences between individual mean values. The unpaired Student *t* test was used to compare ACE expression in patients with SR and CAF as well as amounts of activated Erk1/Erk2 in patients with and without ACE inhibitor therapy. Linear regression was used for correlation between the amounts Erk2-mRNA or activated Erk2 and AF duration as well as ACE expression and amounts of activated Erk1/2 and MEK1/2. All values are expressed as mean  $\pm$  SD and a *p* value <0.05 was considered to be statistically significant.

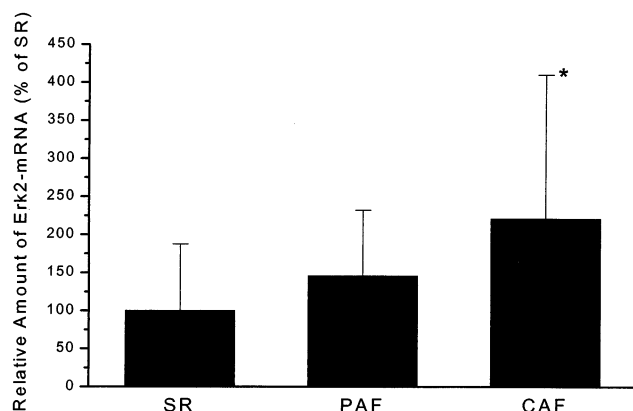
## RESULTS

The baseline patient characteristics are shown in Table 1. The severity of the underlying heart diseases and noncardiac diseases was not different between the groups. The left atrial diameter was increased in patients with CAF compared to patients with SR.

**Erk2 gene expression.** The amount of Erk2 mRNA was significantly increased in patients with CAF (75.8  $\pm$  19.6 U; *n* = 12) compared to patients with SR (30.7  $\pm$  24.9 U; *n* = 12; *p* < 0.05). Patients with PAF had intermediate Erk2 mRNA levels (50.0  $\pm$  29.5 U; *n* = 8). The differences in Erk2 mRNA levels between PAF and patients with SR did not reach statistical significance (Fig. 1). There was no correlation between AF duration and Erk2 mRNA content (*r* = 0.18; *p* = NS) in patients with CAF.

**Phosphorylated Erk1 and Erk2 (Pp44/Pp42).** The tyrosine-phosphorylated forms of Erk1 (Pp44) and Erk2 (Pp42) were significantly increased in atrial tissue of patients with CAF (Erk1: 249  $\pm$  55%; Erk2: 301  $\pm$  48%; *n* = 11; *p* < 0.01) as well as PAF (Erk1: 193  $\pm$  43%; Erk2: 255  $\pm$





**Figure 1.** Atrial expression of Erk2 mRNA in patients with SR (n = 12), in PAF (n = 8) and during CAF (n = 12). Erk2 mRNA was increased in patients with CAF (221 ± 18%). The increase of Erk2 mRNA levels in PAF was not significant (146 ± 86% compared with SR). **Asterisk:** p < 0.05 versus SR.

52%; n = 8; p < 0.01) compared to patients in SR (Erk1: 100 ± 21%; Erk2: 100 ± 22%; n = 11) (Fig. 2). The differences between CAF and PAF were not significant. Similar to the expression of Erk2 mRNA, amounts of activated Erk2 did not correlate with AF duration (r = 0.2; p = NS). Both the amounts of activated Erk1 and Erk2 were reduced in patients with SR treated with ACE inhibitors at the time of surgery (Erk2: 158 ± 46 U; n = 9 vs. 334 ± 67 U; n = 7; p < 0.02; Erk1: 58 ± 61 U vs. 135 ± 63 U; p < 0.05) (Fig. 3). Linear regression analysis showed a trend toward an inverse relation between ACE expression and the amount of activated Erk1/2 (r = -0.3; p = NS) and between ACE expression and the amount of activated MEK1/2 (r = -0.3; p = NS) in this group of patients. There were no other differences in clinical parameters (age, ejection fraction, severity of heart disease, etc.) among these 16 patients with and without ACE inhibitors. Reduced Erk2 activity was also found in patients with CAF who were treated with ACE inhibitors (234 ± 79 U; n = 9 vs. 464 ± 279 U; n = 7; p < 0.05). Slight changes in the amounts of activated Erk1 did not reach statistical significance (73 ± 29 U vs. 125 ± 87 U; p = NS). No linear relation between ACE expression and activated Erk1/2 could be demonstrated in patients with CAF (r = 0.2; p = NS).

**Expression of Erk-activating kinases (MEK1/2).** The relative amount of activated (phosphorylated) Erk-activating kinases (MEK1/2) was increased during CAF (MEK1/2: 168 ± 21%; n = 11; p < 0.05) as well as PAF (MEK1/2: 176 ± 14%; n = 8; p < 0.05) compared with SR (100 ± 7%; n = 11). Comparable to the amounts of activated Erk1/Erk2, the differences in the amounts of activated MEK1/2 in patients with CAF or PAF were not significant either (Fig. 2).

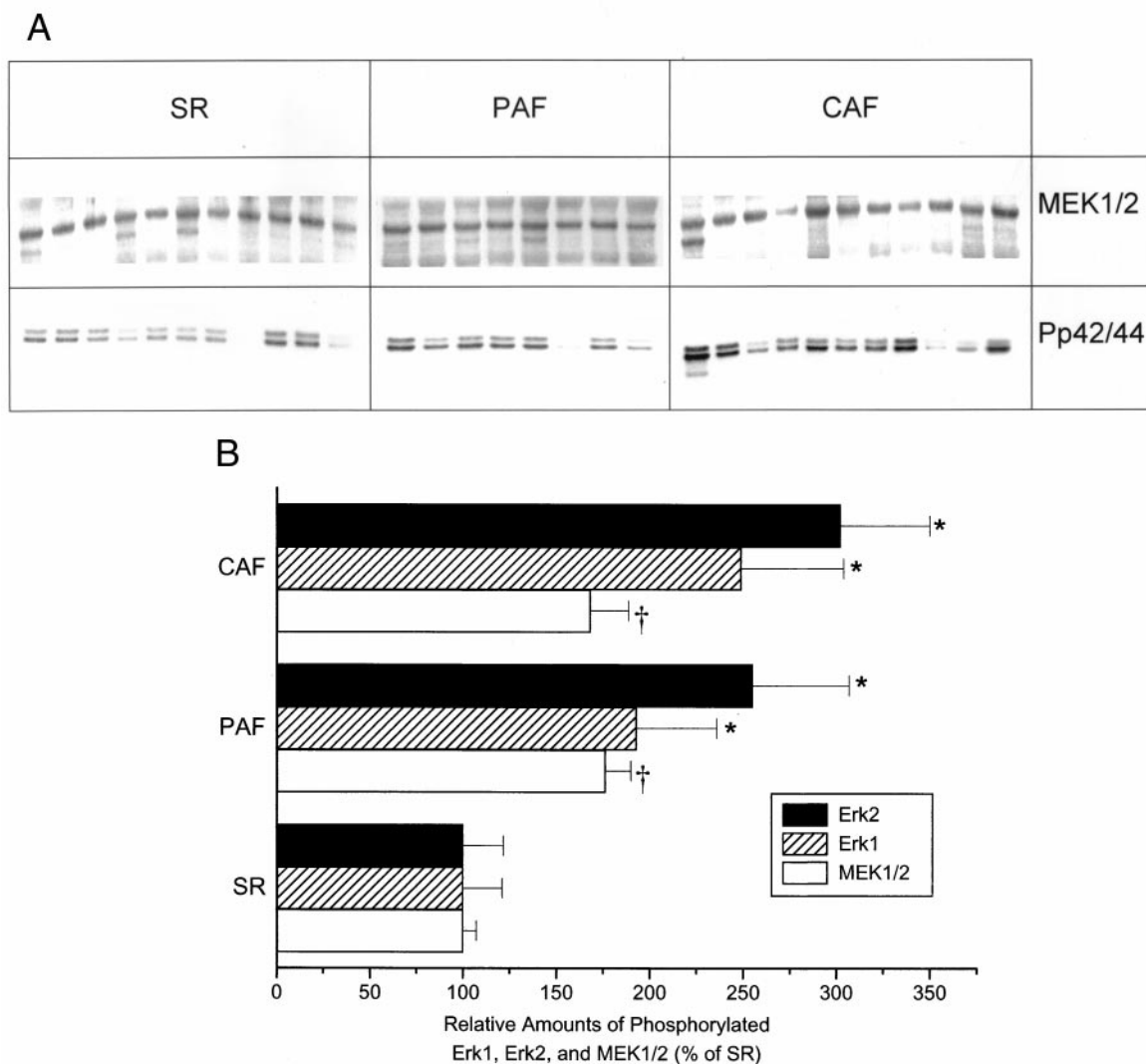
**Expression of ACE.** The expression of ACE was determined in seven patients with CAF and in seven matched patients with SR. None of the patients had been treated with ACE inhibitors before surgery. The expression of ACE was found to be substantially increased in patients with CAF (ACE: 297 ± 44%; p < 0.01) compared with patients in SR (Fig. 4). The expression of ACE correlated with the amount of activated Erk1 (r = 0.6; p = 0.06) and Erk2 (r = 0.4; p = NS) in patients with CAF. The correlation between ACE expression and activated MEK1/2 was r = 0.3 (p = NS). Similarly, only weak linear relations between ACE expression and amounts of activated Erk1/2 (r = 0.4; p = NS) as well as ACE expression and activated MEK1/2 (r = 0.3; p = NS) were found in patients with SR.

**Histochemistry and immunohistochemistry.** The immunohistochemical distribution of activated Erk1/Erk2 in the atria showed similar staining patterns in patients with (n = 12) or without AF (n = 10): Erk1/Erk2 was found in the cytoplasm of interstitial cells (most likely representing fibroblasts), vascular endothelial cells and pericardial fat cells. The overall number of immunoreactive cells, particularly of interstitial cells (Fig. 5), and the intensity of immunostaining appeared to be increased in the atria of patients with AF compared to those without AF. No significant difference was found between PAF and CAF. Few atrial myocytes were immunoreactive for Erk1/2 only in two cases of CAF (one patient with coronary artery disease, one patient with aortic valve disease). However, antigenicity of intracellular proteins was not affected by the fixation procedure as demonstrated by intense immunostaining of myocytes with antidesmin (not shown) or antimyoglobin (Fig. 5C).

Extensive interstitial fibrosis, evidenced by a Masson trichrome stain, was found in patients with AF (Fig. 5D). The amount of fibrosis exceeded impressively the degree of fibrosis seen in patients without AF.

## DISCUSSION

**Main findings.** To the best of our knowledge, this study discusses for the first time a molecular mechanism being responsible for the development of atrial fibrosis in patients with AF. Atrial tissue of patients with CAF or PAF showed increased levels of phosphorylated (activated) Erk1 and Erk2. Increased amounts of phosphorylated Erk1 and Erk2 presumably result from an increased activity of Erk-activating kinases MEK1/2. The amounts of activated Erk1/Erk2 were not related to the arrhythmia duration as evidenced by similar levels of phosphorylated Erk1/Erk2 in patients with CAF and PAF. Besides the enhanced levels of activated Erk2, the expression of Erk2 mRNA and the expression of ACE were increased during CAF. The amount of activated Erk2 was reduced in patients who were treated with ACE inhibitors. Immunohistologic study showed activated Erk1/Erk2 predominantly in interstitial cells, the capillary endothelium and in the atrial fat tissue



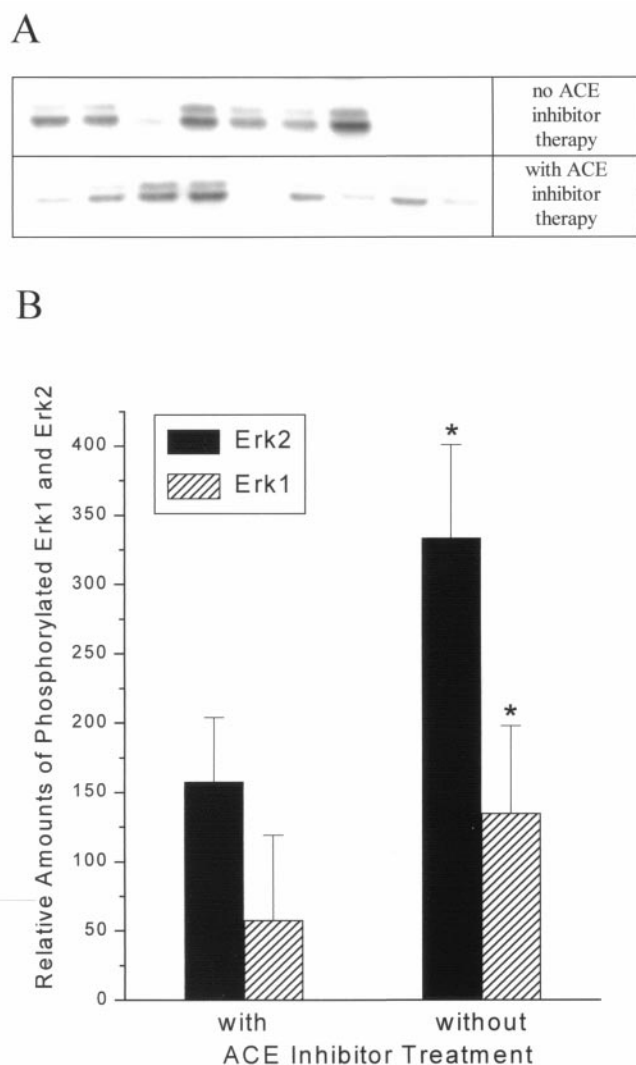
**Figure 2.** A, Western blots demonstrating atrial expression of phosphorylated Erk-activating kinases (MEK1/2) and phosphorylated Erk1/Pp44 (**upper band**) and Erk2/Pp42 (**lower band**) in patients with SR (n = 11), with PAF; (n = 8) and with CAF (n = 11). B, Densitometric quantification of Western blots shown in A. MEK1/2, Erk1 and Erk2 activities are shown as relative densitometric absorption units. **Asterisk:** p < 0.01 versus SR. **Dagger:** p < 0.05 versus SR.

regardless of the underlying atrial rhythm. However, the intensity of Erk immunostaining was increased in interstitial cells of patients with AF and these patients showed marked atrial fibrosis.

#### Mechanisms for increased ACE and Erk2 expression.

Growth signals received by cell membranes are transmitted to the nucleus through an intricate network that consists of a complex array of protein-protein interactions generally governed by phosphorylation (11,12). After binding to its G protein-coupled receptor, Ang II activates Erk1/Erk2 through well described signaling pathways (12,13,16,17). During the final step of this cascade, Erk1/Erk2 are phosphorylated by MEK1/2. Phosphorylated Erks stimulate the phosphorylation and activation of transcription factors such as Fos. This triggers the expression of genes encoding contractile, structural and cell-cycle regulatory proteins

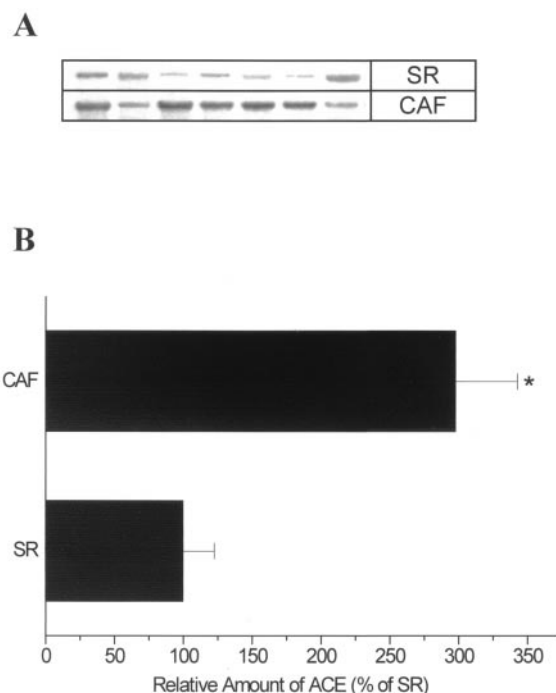
(16,18). Due to these effects, Erk1/Erk2 are known to be involved in cellular growth, proliferation and differentiation processes (11,13,14). Yamazaki et al. (13,16) showed that mechanical stretch can induce cardiac hypertrophy by Ang II- or endothelin I-dependent mechanisms using activation of Erk. They demonstrated in their in vitro experiments that the activity of MAPK increased within minutes by an Ang II-dependent mechanism after mechanical stretch had been applied to cardiac myocytes. In addition to these effects on myocytes, other studies have shown that Ang II can also induce, via activation of MAPK such as Erk1/Erk2, proliferation of fibroblasts as well as extracellular matrix protein accumulation (17,18). It has been reported that the effects of Ang II on cultured myocytes require cardiac fibroblasts in the culture. Purified cardiac myocytes alone did not respond to Ang II (19). These reports support the assumption of a



**Figure 3.** A, Effect of ACE inhibitor therapy on Erk1 (upper band) and Erk2 (lower band) activity during SR. Western blots demonstrating activated Erk1/Erk2 in patients without (n = 7) and with ACE inhibitors (n = 9). B, Densitometric quantification of Western blots shown in A. Amounts of phospho-Erk1 and phospho-Erk2 are shown as relative densitometric absorption units. **Asterisk:**  $p < 0.05$  versus ACE inhibitor treatment.

mechanism whereby Ang II induces the release of fibroblast-derived paracrine factors that trigger the myocardial responses. These findings show that interstitial cells are a main target for Ang II action in cardiac tissue. Some of the Ang II-induced effects on the interstitium could be reversed or blunted by inhibition of the cardiac angiotensin system (18,20).

Our results indicate that CAF and PAF are indeed accompanied by increased atrial expression of ACE and Erk2 as well as elevated levels of activated Erk1/Erk2 and MEK1/2 in humans. The demonstrated activation of the MEK-Erk pathway within the atrial interstitium appears as one molecular mechanism explaining fibrotic changes of the

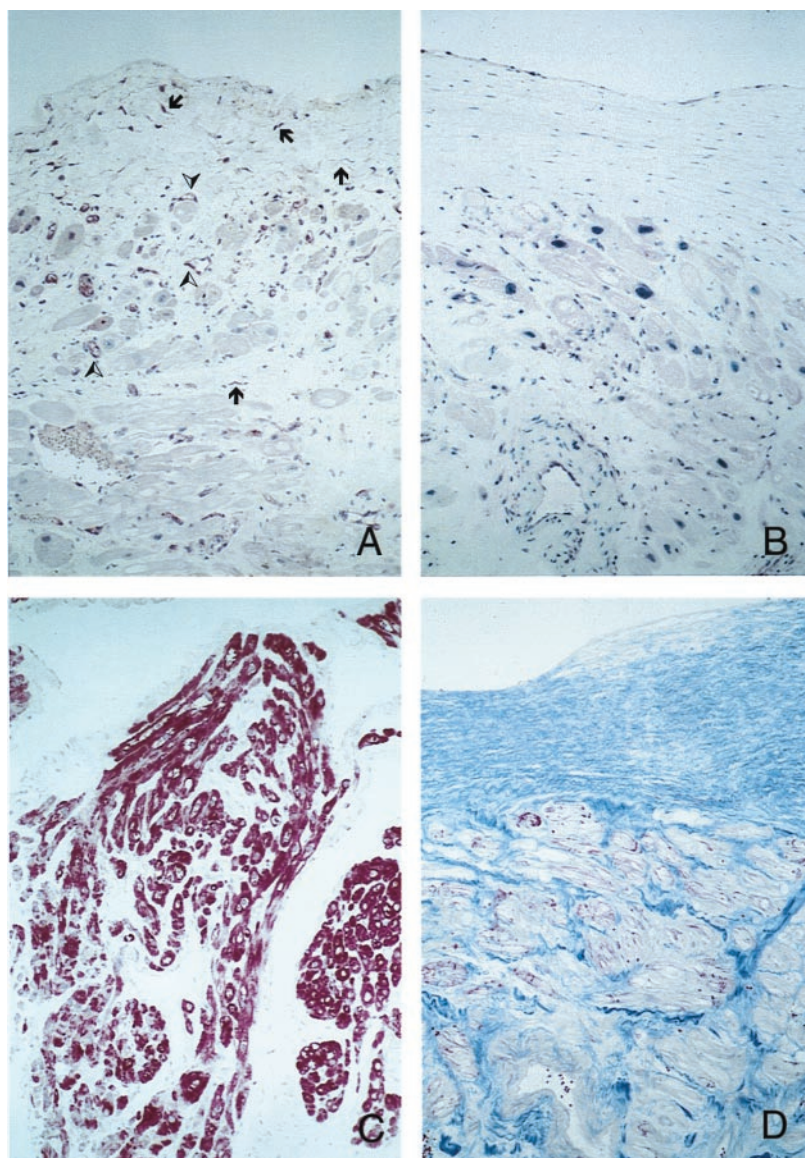


**Figure 4.** A, Western blots demonstrating differences in ACE expression between patients with SR (n = 7) and CAF (n = 7). No patient had received ACE inhibitors prior to surgery. B, Densitometric quantification of the Western blots shown in A. The ACE expression is shown as relative densitometric absorption units. **Asterisk:**  $p < 0.01$  versus SR.

atrial tissue in patients with AF. Within the intact atria, it seems indicative that conditions associated with increased atrial volume and/or filling pressures enhance atrial stretch and thereby trigger the expression of ACE. This may stimulate, via Ang II, the activation of Erk1/Erk2 within interstitial cells. White *et al.* (21) showed that induced AF is accompanied by a substantial increase (up to 80%) in atrial pressure. In addition, it is well known that persistent AF, as found in our patient population, is accompanied by a significant dilation of the atria (3,22). Increased atrial stretch during SR in the presence of various cardiac diseases, however, could cause activation of the Erk cascade and may induce changes in atrial refractoriness, thereby facilitating atrial arrhythmias (23-25). Thus, activation of Erk1/Erk2 may occur prior to or simultaneously with the development of AF in the setting of intraatrial pressure overload. In our study, patients with PAF had similar levels of activated Erk1 and Erk2 compared to patients with CAF. In addition, there was no correlation between Erk2 mRNA expression and AF duration. One can hypothesize, therefore, that increased activation of Erk1/Erk2 induces atrial fibrosis and thereby provides the pathophysiologic substrate that predisposes to the occurrence of AF.

Other signaling pathways may also contribute to the development of structural atrial changes during AF. Particularly, stress-responsive kinases such as cJNK or p38-





**Figure 5.** **A**, Immunostaining of atrial tissue with Erk1/Erk2 antibody in a patient with CAF. Erk1/Erk2 were found within interstitial cells (**arrows**) and vascular endothelial cells (**arrowhead**; magnification  $\times 120$ , reduced by 65%). **B**, Immunostaining with Erk1/Erk2 antibody in a patient with SR. Significantly less immunostaining was found within the tissue specimen (magnification,  $\times 120$ , reduced by 65%). **C**, Intense intracellular immunostaining of myocytes with myoglobin antibody shows that reduced or missing intracellular immunostaining with Erk1/Erk2 is not related to fixation artifacts (same specimen as in **A**; magnification  $\times 120$ , reduced by 65%). **D**, Masson trichrome stain in a patient with CAF. A prominent pericardial fibrotic layer (blue stain) extends from the pericardial surface deep into the atrial interstitium. In addition, interstitial fibrosis is present coating individual myocytes (magnification,  $\times 120$ , reduced by 65%).

MAPK are associated with fibrogenesis (26–28). cJNK and p38-MAPK are activated, for example, in response to inflammatory cytokines, hypoxia, UV irradiation and Ang II (26,27,29). Thus, Ang II may activate several intracellular signal transduction pathways during AF, whereas cytokines or adenosine triphosphate depletion seem not to be responsible for structural changes in fibrillating atria (5,30). Activation of the Erk pathway alone might not be sufficient to induce the full spectrum of fibrotic changes (28,31,32). However, proliferation of fibroblasts, which is an elemental feature in the development of cardiac fibrosis, is dependent

on the MEK-Erk cascade (11,33,34). But, the simultaneous employment and cross-talk of parallel pathways may explain the weak linear correlation between the expression of ACE, MEK1/2, and Erk1/2.

In addition, alterations of Ang II-receptor expression may occur during AF and modulate MAPK activity and expression. An overexpression of the AT1-receptor (35–37) could cause an amplification of the Ang II-dependent signaling. Down-regulation of the AT1-receptor or up-regulation of the AT2-receptor, as seen in patients with terminal ventricular failure, could have an inhibitory effect

on the Erk-cascade (14,38,39). An up-regulation of AT2-receptors due to an increased atrial pressure was described in humans (40). Masaki *et al.* (41) ascertained that AT2-receptor activation decreases the AT1-receptor mediated ERK-activity by activation of ERK-phosphate 1. An increased expression of AT2-receptor, therefore, may inhibit the progression of interstitial fibrosis by decreasing Ang II-induced proliferation of fibroblasts and matrix protein accumulation (14,42). However, despite the demonstrated down-regulation of AT1-receptors in failing hearts, recent studies have also shown that the amounts of phosphorylated Erk1/2 are not decreased in this setting (43,44). Further studies are warranted to elucidate the regulation of Ang II-receptor expression in patients with AF.

**Atrial fibrosis and AF.** Atrial fibrillation causes significant electrophysiologic and structural changes in the atrial tissue (3–6). Several studies have demonstrated that prolonged episodes of AF are accompanied by a “tachycardia-induced atrial myopathy” (3,6,10). In contrast to animal models of pacing-induced AF, atrial biopsy specimens from patients with persistent AF revealed interstitial fibrosis and vascular degeneration of atrial myocytes (8). In the present study, patients with AF showed atrial fibrosis and dilated atria that have been reported by others (3,10,22). Whether all fibrotic alterations of the atrial tissue are induced by AF itself is not known. Li *et al.* (9) used a canine model to show that pacing induced heart failure is associated with the development of atrial fibrosis. In this model, atrial fibrosis resulted in increased conduction heterogeneity favoring the inducibility of AF. Atrial refractoriness, however, showed no changes. Similarly to the animal experiments, spatial inhomogeneities of conduction were also demonstrated in the right and left atria from patients with AF and mitral valve disease (45). The hypothesis that atrial fibrotic changes are already present in early phases of AF is supported by our findings that tissue specimens from patients with PAF and CAF showed similar amounts of activated Erk1/Erk2. Persistently increased Erk activity over years during CAF, however, may cause progressive atrial fibrosis. The time period of increased Erk activity and the amount of fibrotic tissue may determine the difference between paroxysmal and persistent AF.

**Clinical implications.** The present study provides evidence that AF is associated with an increased expression of ACE and increased amounts of activated Erk1/Erk2 in atrial tissue. We could show that the level of an activated Erk1/Erk2 is lower during treatment with ACE inhibitors. It is of great interest to show in prospective studies whether ACE inhibitor treatment influences the clinical course as well as the morphologic appearance of the atria in patients with AF. This is supported by results from Van Den Berg *et al.* (46) who showed a beneficial effect of ACE inhibitor treatment in patients with AF and heart failure. Furthermore, a clinical trial demonstrated that ACE inhibitor therapy reduces the incidence of AF in patients with left

ventricular dysfunction after a myocardial infarction (47), a finding that might be explained by an inhibition of the Erk pathway as demonstrated in the present study.

**Study limitations.** Some potential limitations may have influenced our results. All patients included in our study underwent cardiac surgery and had coronary artery disease or significant valve diseases. The pathophysiologic mechanism of AF in lone AF patients may be different. Different cardiac diseases, however, were distributed similarly within the analyzed groups. The underlying heart disease had no obvious impact on Erk1/Erk2 expression or activation. In contrast to autopsy and animal studies, we examined only tissue specimens from the right atrial appendage. Thus, no comment can be made regarding the distribution of changes in activated Erk1/Erk2 within the rest of the atria. cJun and p38-MAPK expression was not determined. Increased expression of these MAPKs may contribute to the development of atrial fibrosis during AF. Further studies are needed to determine the impact of AF on atrial cJun and p38-MAPK activity. Ang II tissue levels were not determined in the study. The described increased expression of ACE, therefore, may not necessarily result in enhanced Ang II activity. The amount of activated Erk1/2, however, was related to ACE expression. The extent of fibrosis was not quantified. However, the differences in atrial fibrosis between patients with and without AF were strikingly evident.

## CONCLUSIONS

To the best of our knowledge, the present study demonstrates for the first time an increase in atrial expression and activation of Erk1/Erk2 in patients with AF. Enhanced activation of Erk1/Erk2 within interstitial cells, most likely due to an activated angiotensin system, may explain the presence of marked atrial fibrosis in association with AF. These findings may have an important impact on the treatment of AF with ACE inhibitors.

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